American Journal of Transplantation 2012; 12: 1157–1167
Wiley Periodicals Inc.

doi: 10.1111/j.1600-6143.2012.04013.x

Evolution and Clinical Pathologic Correlations of *De Novo* Donor-Specific HLA Antibody Post Kidney Transplant

C. Wiebe[a,†], I. W. Gibson[b,c,†], T. D. Blydt-Hansen[d], M. Karpinski[e], J. Ho[e], L. J. Storsley[e], A. Goldberg[d], P. E. Birk[d], D. N. Rush[e] and P. W. Nickerson[a,c,*]

[a]*Department of Medicine and Immunology,* [b]*Department of Pathology, University of Manitoba, Canada*
[c]*Diagnostic Services of Manitoba, Canada*
[d]*Department of Pediatric and Child Health,* [e]*Department of Medicine, University of Manitoba, Canada*
**Corresponding author: Peter W. Nickerson, pnickerson@hsc.mb.ca*
†*Both the authors contributed equally.*

The natural history for patients with *de novo* donor-specific antibodies (dnDSA) and the risk factors for its development have not been well defined. Furthermore, clinical and histologic correlation with serologic data is limited. We studied 315 consecutive renal transplants without pretransplant DSA, with a mean follow-up of 6.2 ± 2.9 years. Protocol (n = 215) and for cause (n = 163) biopsies were analyzed. Solid phase assays were used to screen for dnDSA posttransplant. A total of 47 out of 315 (15%) patients developed dnDSA at a mean of 4.6 ± 3.0 years posttransplant. Independent predictors of dnDSA were HLA-DRβ 1 MM > 0 (OR 5.66, p < 0.006); and nonadherence (OR 8.75, p < 0.001); with a strong trend toward clinical rejection episodes preceding dnDSA (OR 1.57 per rejection episode, p = 0.061). The median 10-year graft survival for those with dnDSA was lower than the No dnDSA group (57% vs. 96%, p < 0.0001). Pathology consistent with antibody-mediated injury can occur and progress in patients with dnDSA in the absence of graft dysfunction and furthermore, nonadherence and cellular rejection contribute to dnDSA development and progression to graft loss.

Key words: Antibody-mediated rejection, donor-specific antibody, kidney transplant, transplant rejection, subclinical AMR, nonadherence

Abbreviations: cPRA, calculated panel reactive antibody; Cr, creatinine; dnDSA, *de novo* donor-specific antibody; ELISA, enzyme-linked immunosorbent assay; HLA, human leukocyte antigen; IFTA, Interstitial fibrosis and tubular atrophy; MM, mismatches.

Received 12 October 2011, revised 29 November 2011 and accepted for publication 22 December 2011

Introduction

The development of *de novo* donor-specific HLA antibodies (dnDSA) posttransplantation has been associated with higher graft failure rates (1–10). Moreover, dnDSA can appear before graft loss suggesting that dnDSA may represent a mechanism of repetitive injury and a potential prognostic biomarker (2,11–13). Unfortunately, early studies of *de novo* antibodies employed less sensitive and accurate cytotoxicity or ELISA assays, which may have misclassified patients, and often did not determine the donor specificity of the *de novo* HLA antibody, which has recently been shown to be important (6,7). In addition, most studies evaluate sera for HLA antibodies at a single time point with no serial evaluation to determine when they develop and if they persist at the time of graft failure. Furthermore, the true importance of dnDSA may be underestimated as they frequently appear late posttransplant, whereas many studies have focused serum antibody assessment on the early posttransplant period (i.e. first 5 years) (14–16).

Importantly, there has been limited correlation of clinical and serologic events with histopathology in patients with dnDSA to infer causal relationships (17). Even the most comprehensive pathologic analysis by Hidalgo et al. examined patients with graft dysfunction and therefore was unable to determine the frequency and timing of dnDSA onset relative to the onset of graft dysfunction and had no information as to the impact of dnDSA occurring in the absence of graft dysfunction (7). Because clinical status at the time of dnDSA development may vary from normal function to acute dysfunction with rapid graft loss, the relevance of dnDSA across this spectrum warrants further study.

This study describes the sequential evaluation of sera for dnDSA in a consecutive cohort of kidney transplants from a single center utilizing the most sensitive HLA antibody detection techniques. The risk factors for dnDSA development, the correlation of dnDSA with clinical and pathologic

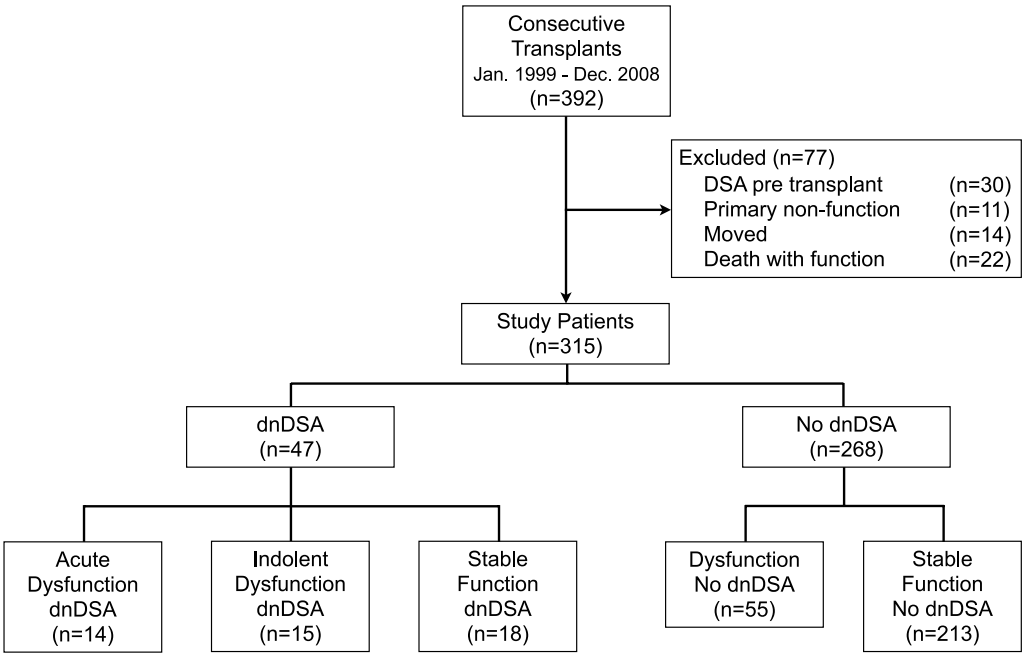

Figure 1: Patient flow.

outcomes and the significance of dnDSA in patients with normal graft function are examined.

Methods

Patient population

Approval was obtained from the Institution Health Research Board (H2011: 211). Three hundred and ninety-two consecutive patients received renal transplants at the Health Sciences Centre, Winnipeg, Manitoba between January 1999 and December 2008. Seventy-seven patients were excluded (pretransplant DSA n = 30), death with a functioning graft (n = 22), moved and lost to follow-up (n = 14), primary nonfunction (n = 11), leaving 315 patients (adult n = 270, pediatric n = 45) for analysis (Figure 1). This cohort was largely Caucasian (72%) but also included Aboriginal (16%), Asian (8%) or African-American (2%) patients. Standard immunosuppression consisted of a calcineurin inhibitor (tacrolimus [n = 249] or cyclosporin [n = 65]), an antiproliferative (mycophenolate mofetil [n = 313] or azathioprine [n = 1]) and prednisone (n = 314). Induction therapy with thymoglobulin (n = 30) or basiliximab (n = 68) was used in 98/315 (31%) of patients. There was one transplant recipient with an HLA identical twin living donor who was not on immunosuppressive medication.

Antibody monitoring

Serum samples were collected and stored at 0,1, 2, 3, 6, 12, 18 and 24 months, then yearly, or at the time of biopsy for graft dysfunction, as routine clinical practice in our program since 1990 (Figure 2). Since 2007 posttransplant surveillance for dnDSA was instituted for all renal transplant patients. DSA screening was performed using FlowPRA beads representing HLA-A, -B, -Cw, -DR, -DQ and -DP antigens (One Lambda, Canoga Park, CA, USA). If the screening assay was positive, determination of HLA antibody specificities was performed using FlowPRA single antigen class I and II beads (One Lambda) and analyzed according to the manufacture's recommendations. HLA antibody specificities were validated using LABScreen single antigen beads using a threshold mean fluorescence intensity ≥300 (One Lambda).

Pretransplant all patients had remote and immediate pretransplant sera screened by FlowPRA and if positive evaluated by FlowPRA single antigen beads. Even if the FlowPRA screen was negative, patient sera were still evaluated by FlowPRA single antigen beads if there was elevated risk of sensitization (e.g. pregnancy, history of transfusion). To rule out a DSA pretransplant, the mismatched donor antigens had to be represented on the single antigen beads. If donor-specific antibodies were absent pretransplant, as determined by solid phase assays and a negative flow cross-match, and became detectable posttransplant they were classified as dnDSA. Patients with dnDSA had banked posttransplant serum tested to determine the approximate timing of dnDSA onset by FlowPRA single antigen beads. All patients continue to be prospectively tested for dnDSA according to the serum collection schedule outlined above to detect new dnDSA or to assess the persistence of existing dnDSA.

Clinical and pathologic monitoring

Study patients were followed at a single center in the adult or pediatric transplant clinic. Protocols beyond 6 months include serum creatinine (Cr) measurement every 4–8 weeks, and quarterly urine collections for proteinuria assessment. Six-month protocol biopsies were performed on all consenting patients (Figure 2). Renal biopsy was offered to all newly detected dnDSA patients since January 2008 as standard of care. Clinically indicated allograft biopsies were performed if proteinuria was ≥0.5 g/day or the serum Cr rose ≥25% from baseline without a known cause. Clinical rejections were biopsied proven in 92%, including 100% of the clinical rejections preceding the onset of dnDSA in the dnDSA subgroup. Biopsies were evaluated using the Banff criteria by a single pathologist who was blinded to DSA status in most but not all cases (18).

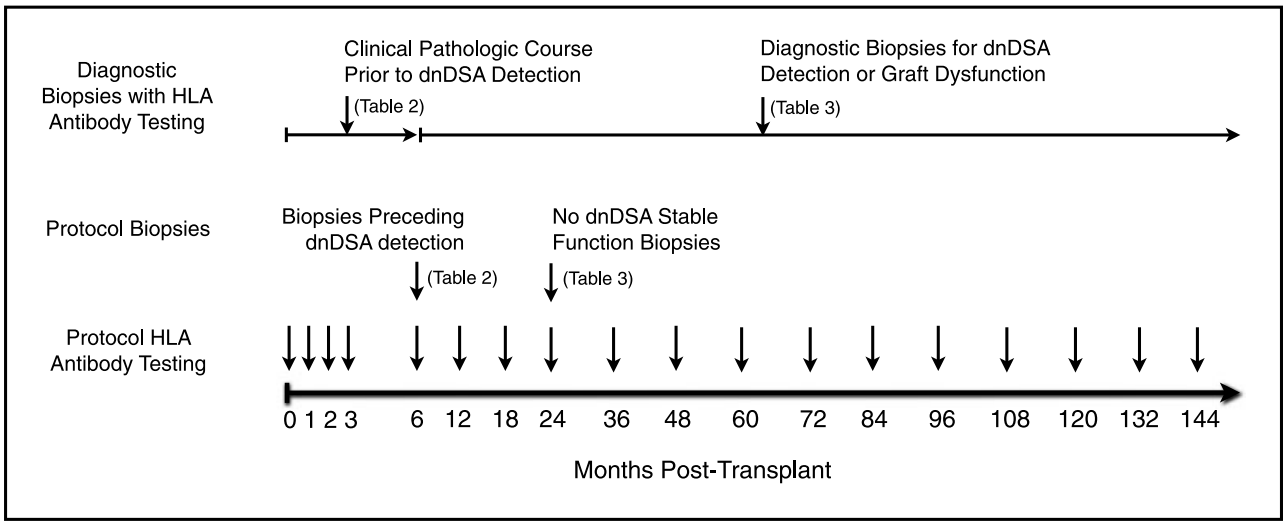

Figure 2: Study design.

Clinical classification of patient phenotypes at the time of dnDSA detection

Patients were classified into five phenotypes based on clinical presentation at the time dnDSA was first detected. Patients in the "Acute Dysfunction dnDSA" group were those with dnDSA and an acute rise in Cr ≥ 25% from baseline in ≤2 months (n = 14). The "Indolent Dysfunction dnDSA" group were patients with dnDSA and graft dysfunction (proteinuria ≥0.5 g/day or Cr ≥ 25% baseline) whose Cr increased by 25% in >2 months (n = 15). The group "Stable Function dnDSA" had dnDSA detected by routine surveillance but had no graft dysfunction (proteinuria ≥0.5 g/day or Cr ≥ 25% baseline, n = 18). Patients with no dnDSA who had persistent graft dysfunction (proteinuria ≥0.5 g/day or Cr ≥25% baseline) were categorized as "Dysfunction No DSA" (n = 55), and 35 of 55 were biopsied. Finally, the "Stable Function No DSA" group had neither dnDSA nor persistent graft dysfunction (n = 213); 27 of these patients had 24 month protocol biopsies as part of a previous study and have now remained free of dysfunction for ≥5 years, thus serving as a histological control group (19).

Nonadherence was defined as patient admission of medication nonadherence documented by clinic staff and/or drug levels below the detectable limit. Repeated failure to attend clinic visits or perform laboratory evaluations (i.e. blood draws for medication levels) constituted a pattern of behavior defined as nonadherence in a minority of patients.

Statistical analysis

Comparisons between baseline predictors and clinical outcomes were done using Student's *t*-test for parametric continuous variables and Wilcoxon signed-rank test for nonparametric data. Chi-squared or Fisher's exact tests were used to test categorical variables. Survival analysis was done by the Kaplan–Meier method using the log-rank test for significance. Linear regression analysis was used to determine the association between two continuous variables. Nominal logistic regression analysis using a step-wise approach was performed to search for significant predictors of dnDSA and graft loss. All univariate correlations with a p-value <0.2 were initially considered in the models.

Results

Patient outcomes and risk factors for* de novo *DSA and graft loss

Overall the entire patient cohort represented a low-risk group with 97% receiving their first transplant and 90% of patients having a calculated panel reactive antibody (cPRA) <10%. However, posttransplant antibody surveillance during a mean follow-up of 6.2 ± 2.9 years found that 47 of 315 (15%) patients developed dnDSA. The mean time from transplantation to development of dnDSA was 4.6 ± 3.0 years. No patient had dnDSA before 6 months posttransplant. Thirty-two of 47 patients with dnDSA had class II antibodies alone, 3 had class I alone and 12 had both class I and II dnDSA. Once present the dnDSA persisted, or in some cases underwent epitope expansion (Table A1). An additional 18% of patients formed *de novo* HLA antibody that was not donor-specific (class I, n = 38; class II, n = 13; classes I and II, n = 5).

The 10-year graft survival for patients with dnDSA was lower than that of the no dnDSA group (59% vs. 96%, p < 0.0001, Figure 3A). There was no difference in 10-year graft survival between the group with *de novo* HLA antibodies, pretransplant HLA antibodies (n = 39), and those with no antibodies (p = 0.817, Figure 3B). Patients with dnDSA had a nonsignificant trend toward a worse 10-year graft survival (p = 0.197, Figure 3C) compared with patients with graft dysfunction from other causes.

Patients in the dnDSA group were younger (33 years vs. 42 years, p = 0.008, Table 1) and had longer cold ischemic times (8.9 h vs. 7.5 h, p = 0.020) than those with no dnDSA. The dnDSA group had a higher number of total HLA

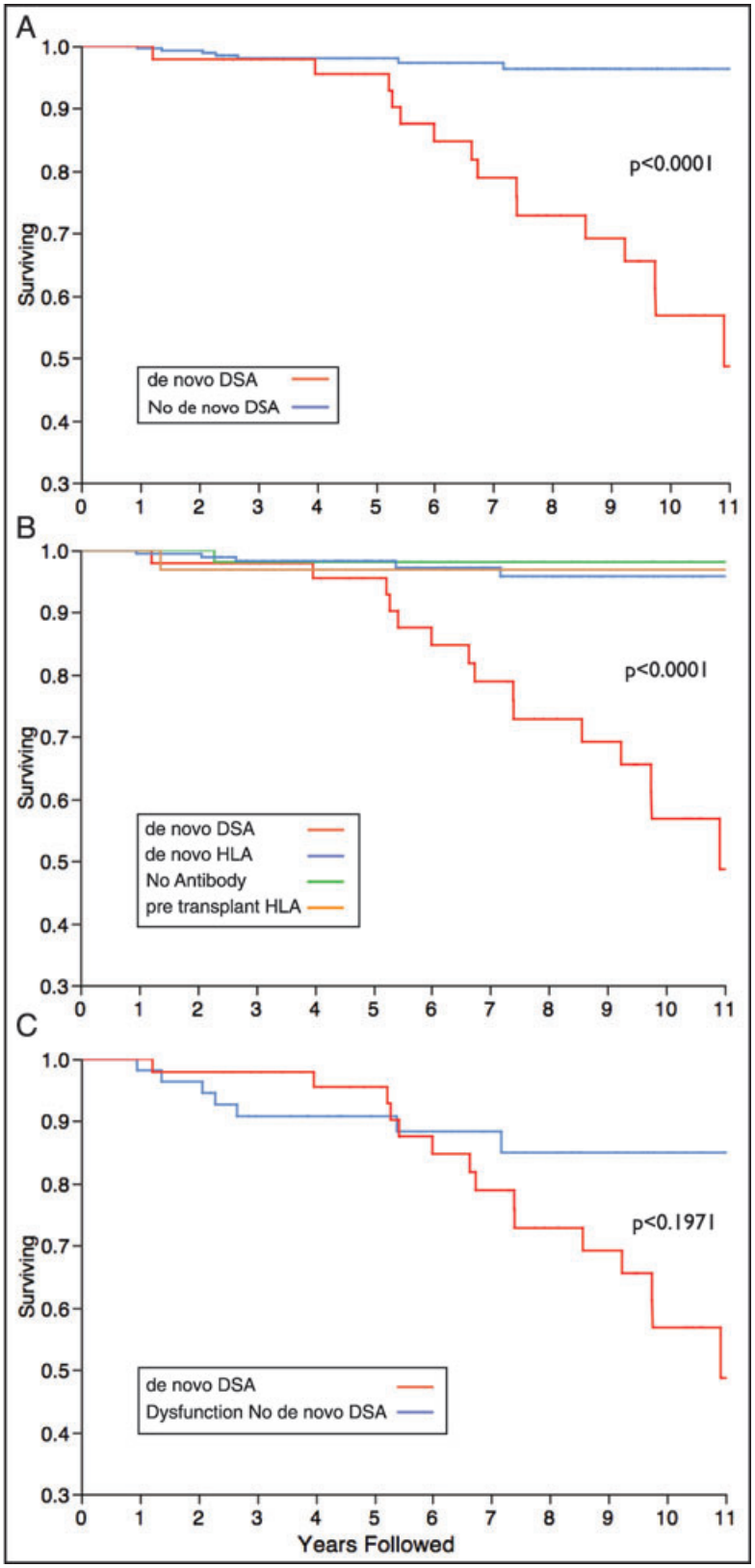

Figure 3: Kaplan–Meier estimates of graft survival. (A) The graft survival of patients with *de novo* donor-specific antibodies (dnDSA) versus those without. (B) The graft survival of pretransplant human leukocyte antibodies (HLA) antibodies, posttransplant *de novo* HLA antibodies, or no antibodies compared to patients with dnDSA. (C) The graft survival of those with dnDSA compared to those with dysfunction from other causes.

Table 1: Baseline characteristics

	dnDSA (n = 47)	No dnDSA (n = 268)	p-Value
First transplant	96%	98%	0.555
Adult recipient	72%	88%	0.009
Recipient age (years)	33 ± 17	42 ± 16	0.008
Donor age (years)	36 ± 15	40 ± 14	0.053
Living donor	43%	53%	0.211
cPRA%	3.4 ± 14	5.3 ± 17	0.371
HLA-A MM	1.04 ± 0.6	0.97 ± 0.7	0.469
HLA-B MM	1.17 ± 0.6	1.08 ± 0.7	0.432
HLA-DRβ1 MM	1.15 ± 0.5	0.87 ± 0.7	0.005
HLA-DRβ3 MM	0.26 ± 0.4	0.16 ± 0.4	0.097
HLA-DRβ4 MM	0.21 ± 0.4	0.13 ± 0.3	0.118
HLA-DRβ5 MM	0.15 ± 0.4	0.15 ± 0.4	0.979
HLA-DQβ1 MM	0.96 ± 0.5	0.80 ± 0.7	0.085
Total MM	3.28 ± 0.9	2.84 ± 1.6	0.009
Cold ischemic time (h)	8.9 ± 6	7.5 ± 6	0.020

dnDSA = *de novo* donor-specific antibody; cPRA = calculated panel reactive antibody; HLA = human leucocyte antigen; MM = mismatch.

mismatches (MM) compared to the no dnDSA group (3.28 vs. 2.84, p = 0.009, Table 1) driven largely by HLA-DRβ1 MM (1.15 vs. 0.87, p = 0.005). Other pretransplant risk factors were similar between the two groups.

Clinical pathologic correlations before the onset of dnDSA

There was no difference between the two groups with regard to delayed graft function. Posttransplant there was a large difference in the rate of nonadherence between those with and without dnDSA (49% vs. 8%, p < 0.001, Table 2 %%%). Nonadherence was more prevalent in the pediatric cohort (24% vs. 13%, p = 0.035).

The onset of dnDSA was ≥6 months in all patients (range 6–130). Interestingly, 0–6 month clinical rejection episodes (borderline or Banff 1A/1B cellular rejections) occurred more commonly in the dnDSA group compared with the no dnDSA group (28% vs. 13%, p = 0.015, Table 2). Moreover, despite a median acute glomerulitis (g) score of zero in both groups, the dnDSA group had significantly higher peritubular capillaritis (ptc) scores in 0–6 month clinical rejection biopsies compared to the no dnDSA group (2 vs. 1, p = 0.049). Both the clinical rejection frequency and their ptc scores were higher independent of adherence in the dnDSA patients. Similarly, dnDSA patients had a trend toward more subclinical rejections (Banff 1A or higher) in the first 6 months compared to the no dnDSA group (26% vs. 15%, p = 0.100).

Before the onset of dnDSA, the only significant differences found in the 6-month protocol biopsies between the no dnDSA and the dnDSA groups were the interstitial inflammation (i) (0.37 vs. 0.62, p < 0.05) and ptc (0.11 vs. 0.60, p < 0.01) scores (Table 2). However, a subanalysis of the dnDSA group found that these differences were confined to the nonadherent dnDSA subgroup. Indeed, in the nonadherent dnDSA group 9 of 19 had borderline or Banff 1A/1B subclinical cellular rejections.

A stepwise logistic regression analysis identified two predictors of dnDSA after adjustment with a strong trend for a third: HLA-DRβ1 MM > 0 (OR 5.66, p < 0.006); and nonadherence (OR 8.75, p < 0.001); and clinical rejection episodes preceding dnDSA (OR 1.57 per rejection episode, p = 0.061). After adjustment independent predictors for graft loss included: recipient age (OR 1.06 per year younger, p = 0.005), delayed graft function requiring dialysis (OR 5.21, p = 0.023), clinical rejection episodes preceding dnDSA (OR 1.95 per rejection episode, p = 0.015), nonadherence (OR 4.34, p = 0.016) and dnDSA (OR 6.34, p = 0.004).

Pathologic correlations with patient phenotypes at the time of dnDSA detection

Table 3 summarizes the three different patient phenotypes based on graft function at the time of dnDSA detection. Nonadherence was documented in 100% of the Acute Dysfunction dnDSA group, in 53% of the Indolent Dysfunction dnDSA group and in only 6% of the Stable Function dnDSA groups (p < 0.001). These groups were compared to patients with Stable Function and No dnDSA and to a group with persistent graft dysfunction with no dnDSA.

Acute Dysfunction dnDSA group

In this group, the onset of dnDSA was essentially concurrent with the onset of clinical dysfunction and the mean serum Cr at biopsy was higher than that of the Indolent Dysfunction dnDSA group (490 μmol/L vs. 156 μmol/L, p = 0.016, Table 3). The Acute Dysfunction dnDSA group had higher g (0.92 vs. 0.04, p < 0.001), ptc (2.20 vs. 0.04, p < 0.001), C4d+ (diffuse or focal) (80% vs. 4%, p = 0.001) and cg (0.25 vs. 0, p = 0.008) scores in comparison to the Stable Function No dnDSA group consistent with a diagnosis of acute and

Table 2: Clinical pathologic course before dnDSA detection

	No dnDSA (n = 268)	Total dnDSA (n = 47)	dnDSA adherent subgroup (n = 24)	dnDSA nonadherent subgroup (n = 23)
Non-adherence	8%	49%***	0%	100%
DGF requiring dialysis	12%	11%	8%	13%
Clinical rejection, 0–6 months	13%	28%*	29%*	26%
Subclinical rejection, 0–6 months	15%	26%	30%	22%
6-Month protocol biopsy, n	151	37	18	19
g	0.02 ± 0.2	0.03 ± 0.2	0.05 ± 0.2	0.0 ± 0.0
i	0.37 ± 0.6	0.62 ± 0.8*	0.33 ± 0.6	0.90 ± 0.9**
t	0.41 ± 0.7	0.62 ± 0.9	0.28 ± 0.7	0.95 ± 1.0**
v	0.01 ± 0.1	0.03 ± 0.2	0.06 ± 0.3	0.0 ± 0.0
ptc	0.11 ± 0.4 (n = 46)	0.60 ± 0.9 (n =30)**	0.14 ± 0.5 (n =14)	1.0 ± 1.0 (n =16)**
C4d+	0% (n =16)	10% (n =31)	7% (n =14)	12% (n =17)
cg	0.02 ± 0.2	0.03 ± 0.2	0.05 ± 0.2	0.0 ± 0.0
ci	0.53 ± 0.6	0.57 ± 0.7	0.56 ±0.7	0.58 ± 0.7
ct	0.65 ± 0.6	0.62 ± 0.6	0.61 ± 0.6	0.63 ± 0.6
cv	0.36 ± 0.6	0.36 ± 0.6	0.44 ± 0.7	0.29 ± 0.5
Clinical rejection, 7–12 months	3%	6%	0%	13%*
12-Month serum Cr. (μmol/L)	113 ± 44	116 ± 44	121 ± 44	110 ± 45
dnDSA onset (months)	–	56 ± 36	51 ± 37	60 ± 34
Month proteinuria ≥0.5 g/d	51 ± 40 (n =43)	67 ± 34 (n =25)	70 ± 40 (n =7)	66 ± 33 (n =18)
Month Cr ≥ 25% baseline	34 ± 31 (n =33)	68 ± 31 (n =29)***	79 ± 28 (n =7)***	65 ± 32 (n =22)***

Significance level compared to the No dnDSA group *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

chronic antibody-mediated injury. It should be noted that i, t and ci scores were also significantly higher in the Acute Dysfunction dnDSA group. In fact, 8 of 12 patients met the criteria for Banff 1A/1B cellular rejection and 1 of 12 patients met criteria for borderline cellular rejection.

Indolent Dysfunction dnDSA group

In this group, the onset of dnDSA preceded the onset of clinical dysfunction by an average of 9 months for proteinuria, and 12 months for serum Cr elevation. This group had higher g (0.92 vs. 0.04, $p < 0.001$), ptc (1.92 vs. 0.04, $p < 0.001$), C4d (39% vs. 4%, $p = 0.009$) and cg (0.92 vs. 0.0, $p < 0.001$) scores in comparison to the Stable Function No dnDSA group consistent with acute and chronic antibody mediated injury. The g and ptc scores were not significantly different compared to the Acute Dysfunction group, however, the percentage of biopsies that were C4d positive was higher in the Acute Dysfunction group ($p = 0.047$). Biopsies were less likely to meet the criteria for Banff 1A/1B cellular rejection compared to the Acute Dysfunction group (1/13 vs. 8/12, $p = 0.004$), whereas 5 of 14 had borderline rejection.

Stable Function dnDSA group

Compared to the Stable Function No dnDSA group, the Stable Function dnDSA group had significantly higher ptc (0.93 vs. 0.04, $p < 0.001$), and C4d (57% vs. 4%, $p < 0.001$) scores. By light microscopy there was no evidence of chronic antibody mediated injury. Only 1 of 14 Stable Function dnDSA patients biopsied had Banff 1A/1B cellular rejection and 2 of 14 patients had borderline cellular rejection. Subsequently, patient's baseline immunosuppression was increased by targeting FK trough levels of 8 ± 2 μg/L and MMF trough levels of ≥2.0 μg/L. The first four patients with Stable Function dnDSA and microvascular inflammation were also treated with both pulse steroids and 2 g/kg intravenous immunoglobulin (IVIg) monthly for three cycles followed by repeat biopsy after a median follow-up of 5.5 months (range 4–8 months). In three of these patients, there was a progression in Banff g, ptc, C4d or cg scores (Table 4).

Dysfunction No dnDSA group

Biopsies were performed in 35 of 55 (64%) patients in the Dysfunction No dnDSA group. Recurrent glomerular disease accounted for 16 of 35 (46%), which included focal segmental glomerulosclerosis (n = 3), IgA nephropathy (n = 10) and other (n = 3). Interstitial fibrosis and tubular atrophy (IFTA) with (n = 8) or without (n = 6) cellular rejection occurred in 14/35 (40%). The remainder included acute tubular necrosis secondary to rhabdomyolysis (n = 1) and BK virus nephropathy (n = 2). There was no significant increase in g, ptc, C4d or cg scores compared to the

Table 3: Pathologic correlations with patient phenotypes at the time of dnDSA detection

	Acute dysfunction dnDSA	Indolent dysfunction dnDSA	Stable function dnDSA	Dysfunction no dnDSA	Stable function no dnDSA
n	14	15	18	55	213
Clinical rejection, 0–6 months	36%*	27%*	22%	24%*	10%
Nonadherence	100%***	53%***	6%	16%*	6%
Month dnDSA positive	60 ± 35	61 ± 31	49 ± 31	–	–
Month protein, ≥0.5 g/d	63 ± 38	70 ± 33	–	51 ± 40	–
Month Cr ≥ 25% baseline	63 ± 34	73 ± 28	–	34 ± 31	–
Biopsy, n	12	13	14	35	27
Month of biopsy	63 ± 34	71 ± 26	53 ± 46	27 ± 21	24 ± 2
Creatinine at biopsy	490 ± 420***	156 ± 59***	118 ± 44	189 ± 180**	106 ± 31
g	0.92 ± 0.8***	0.92 ± 0.8***	0.14 ± 0.4	0.20 ± 0.5	0.04 ± 0.2
i	2.0 ± 1.1***	1.07 ± 0.8**	0.50 ± 0.8	0.74 ± 1.0	0.37 ± 0.6
t	2.0 ± 1.0***	0.54 ± 0.5**	0.35 ± 0.6	0.60 ± 0.9**	0.11 ± 0.3
v	0.08 ± 0.3	0 ± 0	0.21 ± 0.8	0.03 ± 0.2	0 ± 0
ptc	2.20 ± 0.7***	1.92 ± 1.0***	0.93 ± 1.0***	0.27 ± 0.6	0.04 ± 0.2
C4d+	80%***	39%**	57%***	0%	4%
cg	0.25 ± 0.5**	0.92 ± 1.2***	0 ± 0	0.14 ± 0.4	0 ± 0
ci	1.17 ± 0.6*	1.62 ± 0.5***	0.50 ± 0.7	1.37 ± 0.7***	0.67 ± 0.6
ct	1.25 ± 0.6	1.85 ± 0.7***	0.93 ± 0.5	1.46 ± 0.6**	0.93 ± 0.6
cv	0.75 ± 0.8	0.78 ± 0.6	0.57 ± 0.7	0.67 ± 0.7	0.41 ± 0.6
Months of follow-up post-dnDSA detection	29 (1–69)	45 (1–88)	19 (0–128)	–	–
Graft failure	57%***	40%***	0%	15%***	0%

Significance level compared to the Stable Function No dnDSA group *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Stable Function No dnDSA group consistent with the lack of an antibody mediated injury in either group.

Long-term graft outcomes

Graft loss occurred in 22 of 315 patients during the study period (Table 3), of which 14 had dnDSA. Biopsy proven causes of graft loss in the No dnDSA group included IFTA with (n = 3) or without (n = 2) cellular rejection, membranoproliferative glomerulonephritis (n = 1), diabetic nephropathy (n = 1) and BK nephropathy (n = 1). In the dnDSA group, biopsy proven causes of graft loss were chronic active antibody-mediated rejection (AMR, n = 10), IFTA with *de novo* membranous nephropathy (n = 1), and IFTA with concomitant recurrent IgA nephropathy (n = 1). Two patients with dnDSA did not have a biopsy at the time of graft loss.

Discussion

The principal findings of this study are that dnDSA develops in 15% of low-risk renal transplant recipients at a mean of 4.6 ± 3.0 years posttransplant and graft survival at 10 years is reduced by 40% in such patients. The independent risk factors for dnDSA development are HLA-DRβ1 MM, nonadherence, and a strong trend toward clinical rejections before dnDSA onset. In particular, dnDSA patients were more likely to have preceding clinical and subclinical cellular rejections in the 0–6 month posttransplant period. Moreover, in those who develop graft dysfunction the dnDSA typically arises before the onset of proteinuria or a rise in creatinine. In nonadherent patients that present with dnDSA and dysfunction, the histology is often a mixed cellular and AMR. Finally, allograft pathology consistent with antibody-mediated injury can occur in patients with dnDSA in the absence of graft dysfunction and the degree of injury can progress in these patients despite augmented immunosuppression.

Evidence for DSA being *de novo* is supported by the fact that in this low-risk patient population all pretransplant sera were negative for DSA using the most sensitive solid phase and flow crossmatch assays, and by late development of dnDSA at or after 6 months.

The decrease in graft survival for patients who develop dnDSA confirms previous reports (1–8). In contrast, we found that the presence of pre- and posttransplant non–donor-specific HLA antibodies had no correlation with graft survival in agreement with some (6,7), but not all (3–5,8,9,11,13,20) studies. In patients with *de novo* HLA antibodies that are not donor specific it has been proposed that graft dysfunction may be due to dnDSA that is undetectable in the sera due to graft absorption (12). However, in this study this is unlikely as 10-year graft survival is >95%

Table 4: Repeat biopsies in stable function dnDSA patients post-IVIg and pulse steroids

Biopsy month	Serum Cr (μmol/L)	g	i	t	v	PTC	C4d	cg	ci	ct	cv
Patient 1											
Protocol (6 months)	98	0	0	0	0	0	n/a	0	0	0	0
Protocol (24 months)	82	0	0	0	0	0	Neg.	0	0	0	0
dnDSA +ve (61 months)	82	0	0	0	3	0	Neg.	0	0	0	1
Post IVIg (66 months)	91	1	1	1	1	2	Neg.	0	1	1	2
Patient 2											
Protocol (6 months)	128	0	0	0	0	0	Neg.	0	1	1	0
dnDSA +ve (27 months)	123	1	0	0	0	2	Pos.	0	0	1	0
Post IVIg (33 months)	125	2	2	1	0	2	Pos.	0	1	0	0
Patient 3											
dnDSA +ve (6 months)	130	0	2	2	0	2	Pos.	0	0	1	1
Post IVIg (14 months)	185	2	2	1	0	2	Neg.	2	2	2	0
Patient 4											
Protocol (3 months)	90	0	0	0	0	0	Neg.	0	0	0	0
dnDSA +ve (15 months)	93	0	1	1	0	2	Pos.	0	0	1	0
Post-IVIg (19 months)	103	0	1	1	0	2	Pos.	0	0	1	0

dnDSA = *de novo* donor-specific antibody; IVIg = intravenous immunoglobulin.

in these patients; only 9% (4/47) who develop detectable dnDSA had preceding *de novo* HLA antibodies; and HLA mismatch did not predict the development of *de novo* HLA antibodies in the absence of detectable dnDSA (data not shown).

HLA–DRβ1 mismatch was an independent predictor of dnDSA, confirming the work of Hourmant et al. (3). Moreover, this study supports the observation that dnDSA are predominantly directed at class II donor HLA mismatches. Although the reason for this is unclear, a number of mechanisms have been postulated (4,5,7,10,13,21).

Previous studies have documented the link between nonadherence and late rejection, graft dysfunction and loss (22,23). In this study, nonadherence differed significantly across the dnDSA subgroups and was a risk factor for both dnDSA and graft loss. The fact that clinical and subclinical rejection episodes occur equally in adherent and nonadherent dnDSA patients in the first 6 months suggests that nonadherent behavior manifests after the early posttransplant period (Table 2). However, the elevated 6-month protocol biopsy Banff i, t and ptc scores in the nonadherent dnDSA subgroup as well as their higher frequency of clinical rejections from 7 to 12 months would suggest that as early as 6 months posttransplant the effects of nonadherence may become apparent. This is consistent with a report by Chisholm et al. who observed declining adherence levels beyond 5 months posttransplant (24).

In dnDSA patients the frequency of clinical and subclinical rejections in the first 6 months is twice that of the no dnDSA patients regardless of whether the dnDSA patient was subsequently categorized as adherent or nonadherent (Table 2). Moreover, the higher ptc scores in the 0–6 month clinical rejection biopsies, a common feature of cellular rejection (25) suggests that patients at risk for dnDSA have preceding cellular rejections with more intense inflammation within the microvasculature. We postulate that cellular rejection with peritubular capillaritis leads to increased HLA expression in the microcirculation thereby increasing the risk of allo-recognition by the recipient B-cell compartment (26).

Once dnDSA was detectable in the serum, the intensity and frequency of concurrent cellular rejection varied in parallel with nonadherence suggesting cellular rejection may be both a result of nonadherence and a contributor to the degree of graft dysfunction observed in dnDSA patients. The Acute and Indolent Dysfunction dnDSA groups did not differ in g and ptc scores, however, they differed in C4d staining and incidence of Banff 1A/1B cellular rejection. This suggests that complement activation and cell-mediated rejection may account for the greater level of

dysfunction observed in the Acute Dysfunction as compared to the Indolent Dysfunction dnDSA group. Finally, patients in the Acute or Indolent Dysfunction dnDSA groups who had either borderline or Banff 1A/1B cellular rejection combined with AMR were more likely to progress to graft loss compared to those with AMR alone ($p = 0.05$).

In patients with stable graft function who have dnDSA, four lines of evidence would support the pathologic potential of dnDSA in this setting. First, in our study, 10 of 14 Stable Function dnDSA patients had pathologic features consistent with antibody-mediated microvascular injury. Einecke et al. emphasized that HLA antibody-mediated microvascular injury was a major correlate of late graft loss in patients with graft dysfunction (17). They also reported basement membrane duplication in the glomerular and/or peritubular capillaries occurred more commonly in patients with dnDSA, which implied as process of repetitive injury and repair. Unfortunately, electron microscopy was not routinely performed on our protocol biopsies—a clear limitation of the study. Second, dnDSA antibody would arise before the onset of graft dysfunction as it did in the Indolent Dysfunction dnDSA group of which 12 of 14 had features of antibody-mediated microvascular injury. Third, histologic progression over time would provide evidence that Stable Function dnDSA patients are unlikely to remain clinically silent. To date we have repeat biopsies on four patients who showed no change in one and histologic progression in three despite augmentation in immunosuppression. Fourth, development of graft dysfunction would support that dnDSA has pathologic potential in Stable Function dnDSA patients. To date only 2 of 18 patients have progressed to graft dysfunction. However, the median follow-up post-dnDSA in the group was only 19 months. A subgroup analysis of the adherent patients in the Indolent Dysfunction dnDSA group showed that the median time between dnDSA detection and an increase in creatinine was 20 months (data not shown). Given that the Stable Function dnDSA group was 94% adherent it is likely that the current follow-up is only now approaching the point when graft dysfunction might be expected. Other factors, not yet assessed, which may contribute to the slower rate of progression in the Stable Function dnDSA group include lower antibody titer, ability to activate complement, differences in immunogenicity or expression of the specific HLA epitopes, and the regulation of antibody responses. Nevertheless, although further natural history studies of this cohort are warranted, the available evidence supports the hypothesis that Stable Function dnDSA patients are at risk for progression to graft dysfunction/loss.

We propose a continuum of antibody-mediated damage based on a model adapted from the primate studies of Smith et al. (Figure 4) (27). Posttransplant dnDSA is preceded by an antibody-free period. It is likely that inflammatory events (e.g. preceding cellular rejection or graft infection) leads to enhanced interferon-γ levels, which upregulate HLA expression on endothelial cells and stimulates B-cell allorecognition and subsequent long-lived plasma cells producing dnDSA (28,29). At this point dnDSA onset may be overlooked without routine posttransplant monitoring of stable grafts. Nevertheless, biopsies in stable grafts with dnDSA generally reveal histologic changes consistent with microvasculature injury. dnDSA binding vascular endothelium is capable of inducing injury through the activation of complement (via C1q) or recruitment of neutrophils, macrophage or natural killer cells via Fc receptors (26). Sustained microvascular inflammation (i.e. glomerulitis, peritubular capillaritis and vasculitis) eventually leads to progressive tissue fibrosis (i.e. transplant glomerulopathy, loss of peritubular capillaries, and IFTA) resulting in graft dysfunction. Although dnDSA targets the microcirculation, there is often concomitant cellular inflammation, especially in the face of nonadherence, that may accelerate the development and severity of graft dysfunction and shorten the time to graft loss.

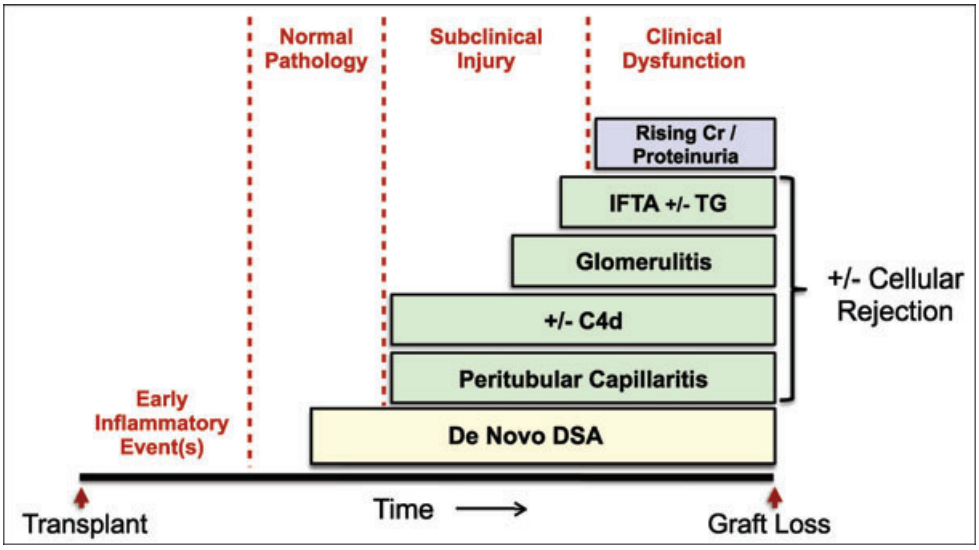

Figure 4: Proposed natural history of dnDSA. This figure shows a proposed model for patients developing *de novo* donor-specific antibodies as they evolve from transplantation to graft failure. IFTA, interstitial fibrosis and tubular atrophy; TG, transplant glomerulopathy. Adapted from Ref.

Limitations of this study include: the time intervals (6 to 12 months) between HLA antibody screening, which may underestimate the time from initial detection of dnDSA to time of graft dysfunction; the separate pediatric and adult cohorts, which may have led to differences in practice confounding the analysis; the assignment of nonadherence may have resulted in false negatives; and the small number of patients in the Stable Function dnDSA group who have had repeat biopsies that may overestimate the risk of progression. Furthermore, because the Stable Function dnDSA patients who had repeat biopsy all received pulse steroids and IVIg we cannot be certain this therapy did not contribute to the observed progression.

Conclusion

To improve long-term outcomes for transplant patients, strategies to prevent and address delayed graft function, cellular rejection and dnDSA associated microvascular injury are required. For example, avoiding HLA-DR MM in allocation, targeting nonadherence early and, avoiding drug minimization protocols in patients at risk of developing dnDSA should be considered. Routine monitoring for dnDSA will identify patients for early interventional studies in an attempt to define effective therapies to alter their prognosis (30). Finally, the importance of cellular rejection should continue to be recognized and treated aggressively given that it frequently precedes dnDSA and itself may be causal in the induction of dnDSA. Moreover, when cellular rejection coincides with dnDSA and antibody mediated microvascular injury it may accelerate the time to graft dysfunction and graft loss.

Acknowledgments

This study was funded by an operating grant from the Canadian Institutes of Health Research. Chris Wiebe is supported by the Manitoba Medical Services Foundation/R. Samuel McLaughlin Fellowship, the Manitoba Health Research Council and the University of Manitoba Department of Medicine. Julie Ho is supported by a KRESCENT new investigator award. Peter Nickerson holds the Flynn Family Chair in Renal Transplantation at the University of Manitoba. This work is possible because of the dedication of the histocompatibility technologists of the Transplant Immunology Laboratory. Statistical assistance was provided by Dr. Depeng Jiang (University of Manitoba, Biostatistical Consulting Unit, Department of Community Health Sciences).

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

References

1. Martin S, Dyer PA, Mallick NP, Gokal R, Harris R, Johnson RW. Posttransplant antidonor lymphocytotoxic antibody production in relation to graft outcome. Transplantation 1987; 44: 50–53.
2. Worthington JE, Martin S, Al-Husseini DM, Dyer PA, Johnson RW. Posttransplantation production of donor HLA-specific antibodies as a predictor of renal transplant outcome. Transplantation 2003; 75: 1034–1040.
3. Hourmant M, Cesbron-Gautier A, Terasaki PI, et al. Frequency and clinical implications of development of donor-specific and non-donor-specific HLA antibodies after kidney transplantation. J Am Soc Nephrol 2005; 16: 2804–2812.
4. Cardarelli F, Pascual M, Tolkoff-Rubin N, et al. Prevalence and significance of anti-HLA and donor-specific antibodies long-term after renal transplantation. Transpl Int 2005; 18: 532–540.
5. Campos EF, Tedesco-Silva H, Machado PG, Franco M, Medina-Pestana JO, Gerbase-DeLima M. Post-transplant anti-HLA class II antibodies as risk factor for late kidney allograft failure. Am J Transplant 2006; 6: 2316–2320.
6. Li X, Ishida H, Yamaguchi Y, Tanabe K. Poor graft outcome in recipients with de novo donor-specific anti-HLA antibodies after living related kidney transplantation. Transpl Int 2008; 21: 1145–1152.
7. Hidalgo LG, Campbell PM, Sis B, Einecke G, Mengel M, Chang J, et al. De novo donor-specific antibody at the time of kidney transplant biopsy associates with microvascular pathology and late graft failure. Am J Transplant 2009; 9: 2532–2541.
8. Terasaki PI, Ozawa M. Predicting kidney graft failure by HLA antibodies: A prospective trial. Am J Transplant 2004; 4: 438–443.
9. Lachmann N, Terasaki PI, Budde K, et al. Anti-Human leukocyte antigen and donor-specific antibodies detected by luminex post-transplant serve as biomarkers for chronic rejection of renal allografts. Transplantation 2009; 87: 1505–1513.
10. Everly MJ, Everly JJ, Arend LJ, et al. Reducing de novo donor-specific antibody levels during acute rejection diminishes renal allograft loss. Am J Transplant 2009; 9: 1063–1071.
11. Lee PC, Terasaki PI, Takemoto SK, et al. All chronic rejection failures of kidney transplants were preceded by the development of HLA antibodies. Transplantation 2002; 74: 1192–1194.
12. Terasaki PI. Humoral theory of transplantation. Am J Transplant 2003; 3: 665–673.
13. Mizutani K, Terasaki P, Rosen A, et al. Serial ten-year follow-up of HLA and MICA antibody production prior to kidney graft failure. Am J Transplant 2005; 5: 2265–2272.
14. Fotheringham J, Angel C, Goodwin J, Harmer AW, McKane WS. Natural history of proteinuria in renal transplant recipients developing de novo human leukocyte antigen antibodies. Transplantation 2011; 91: 991–996.
15. Weinstein D, Braun WE, Cook D, McMahon JT, Myles J, Protiva D. Ultra-Late antibody-mediated rejection 30 years after a living-related renal allograft. Am J Transplant 2005; 5: 2576–2581.
16. Gill JS, Landsberg D, Johnston O, et al. Screening for de novo anti-human leukocyte antigen antibodies in nonsensitized kidney transplant recipients does not predict acute rejection. Transplantation 2010; 89: 178–184.
17. Einecke G, Sis B, Reeve J, et al. Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure. Am J Transplant 2009; 9: 2520–2531.
18. Sis B, Mengel M, Haas M, et al. Banff '09 meeting report: Antibody mediated graft deterioration and implementation of Banff working groups. Am J Transplant 2010; 10: 464–471.
19. Rush D, Arlen D, Boucher A, et al. Lack of benefit of early protocol biopsies in renal transplant patients receiving TAC and MMF: A randomized study. Am J Transplant 2007; 7: 2538–2545.

20. Terasaki PI, Ozawa M, Castro R. Four-Year follow-up of a prospective trial of HLA and MICA antibodies on kidney graft survival. Am J Transplant 2007; 7: 408–415.
21. Sis B, Campbell PM, Mueller T, et al. Transplant glomerulopathy, late antibody-mediated rejection and the ABCD tetrad in kidney allograft biopsies for cause. Am J Transplant 2007; 7: 1743–1752.
22. Vlaminck H, Maes B, Evers G, Verbeke G, Lerut E, Van Damme B, Vanrenterghem Y. Prospective study on late consequences of subclinical non-compliance with immunosuppressive therapy in renal transplant patients. Am J Transplant 2004; 4: 1509–1513.
23. Nevins TE, Kruse L, Skeans MA, Thomas W. The natural history of azathioprine compliance after renal transplantation. Kidney Int 2001; 60: 1565–1570.
24. Chisholm MA, Vollenweider LJ, Mulloy LL, et al. Renal transplant patient compliance with free immunosuppressive medications. Transplantation 2000; 70: 1240–1244.
25. Gibson IW, Gwinner W, Brocker V, et al. Peritubular capillaritis in renal allografts: Prevalence, scoring system, reproducibility, and clinicopathological correlates. Am J Transplant 2008; 8: 819–825.
26. Colvin RB, Smith RN. Antibody-Mediated organ-allograft rejection. Nat Rev Immunol 2005; 5: 807–817.
27. Smith RN, Kawai T, Boskovic S, Nadazdin O, Sachs DH, Cosimi AB, Colvin RB. Four stages and lack of stable accommodation in chronic alloantibody-mediated renal allograft rejection in cynomolgus monkeys. Am J Transplant 2008; 8: 1662–1672.
28. Bishop GA, Hall BM, Suranyi MG, Tiller DJ, Horvath JS, Duggin GG. Expression of HLA antigens on renal tubular cells in culture. I. Evidence that mixed lymphocyte culture supernatants and gamma interferon increase both class I and class II HLA antigens. Transplantation 1986; 42: 671–679.
29. Locke JE, Zachary AA, Warren DS, Segev DL, Houp JA, Montgomery RA, Leffell MS. Proinflammatory events are associated with significant increases in breadth and strength of HLA-specific antibody. Am J Transplant 2009; 9: 2136–2139.
30. Archdeacon P, Chan M, Neuland C, et al. Summary of FDA antibody-mediated rejection workshop. Am J Transplant 2011; 11: 896–906.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1: Donor-specific antibodies detected

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.